This observed effect is of particular utility in assays based on the cleavage of DNA molecules. Using the assays described herein as an example, when an oligonucleotide is shortened through the action of a Cleavase® enzyme or other cleavage agent, the positive charge can be made to not only significantly reduce the net negative charge, but to actually override it, effectively "flipping" the net charge of the labeled entity. This reversal of charge allows the products of target-specific cleavage to be partitioned from uncleaved probe by extremely simple means. For example, the products of cleavage can be made to migrate towards a negative electrode placed at any point in a reaction vessel, for focused detection without gel-based electrophoresis; Example 24 provides examples of devices suitable for focused detection without gel-based electrophoresis. When a slab gel is used, sample wells can be positioned in the center of the gel, so that the cleaved and uncleaved probes can be observed to migrate in opposite directions. Alternatively, a traditional vertical gel can be used, but with the electrodes reversed relative to usual DNA gels (*i.e.*, the positive electrode at the top and the negative electrode at the bottom) so that the cleaved molecules enter the gel, while the uncleaved disperse into the upper reservoir of electrophoresis buffer.

An important benefit of this type of readout is the absolute nature of the partition of products from substrates, *i.e.*, the separation is virtually 100%. This means that an abundance of uncleaved probe can be supplied to drive the hybridization step of the probe-based assay, yet the unconsumed (*i.e.*, unreacted) probe can, in essence, be subtracted from the result to reduce background by virtue of the fact that the unreacted probe will not migrate to the same pole as the specific reaction product.

Through the use of multiple positively charged adducts, synthetic molecules can be constructed with sufficient modification that the normally negatively charged strand is made nearly neutral. When so constructed, the presence or absence of a single phosphate group can mean the difference between a net negative or a net positive charge. This observation has particular utility when one objective is to discriminate between enzymatically generated fragments of DNA, which lack a 3 phosphate, and the products of thermal degradation, which retain a 3 phosphate (and thus two

additional negative charges). Examples 23 and 24 demonstrate the ability to separate positively charged reaction products from a net negatively charged substrate oligonucleotide. As discussed in these examples, oligonucleotides may be transformed from net negative to net positively charged compounds. In Example 24, the positively charged dye, Cy3 was incorporated at the 5' end of a 22-mer (SEQ ID NO:61) which also contained two amino-substituted residues at the 5' end of the oligonucleotide; this oligonucleotide probe carries a net negative charge. After cleavage, which occurred 2 nucleotides into the probe, the following labelled oligonucleotide was released: 5'-Cy3-AminoT-AminoT-3'(as well as the remaining 20 nucleotides of SEQ ID NO:61). This short fragment bears a net positive charge while the reaminder of the cleaved oligonucleotide and the unreacted or input oligonucleotide bear net negative charges.

The present invention contemplates embodiments wherein the specific reaction product produced by any cleavage of any oligonucleotide can be designed to carry a net positive charge while the unreacted probe is charge neutral or carries a net negative charge. The present invention also contemplates embodiments where the released product may be designed to carry a net negative charge while the input nucleic acid carries a net positive charge. Depending on the length of the released product to be detected, positively charged dyes may be incorporated at the one end of the probe and modified bases may be placed along the oligonucleotide such that upon cleavage, the released fragment containing the positively charged dye carries a net positive charge. Amino-modified bases may be used to balance the charge of the released fragment in cases where the presence of the positively charged adduct (*e.g.*, dye) alone is not sufficient to impart a net positive charge on the released fragment. In addition, the phosphate backbone may be replaced with a phosphonate backbone at a level sufficient to impart a net positive charge (this is particularly useful when the sequence of the oligonucleotide is not amenable to the use of amino-substituted bases); Figures 56 and 57 show the structure of short oligonucleotides containing a phosphonate group on the second T residue). An oligonucleotide containing a fully phosphonate-substituted backbone would be charge neutral (absent the presence of modified charged residues

bearing a charge or the presence of a charged adduct) due to the absence of the negatively charged phosphate groups. Phosphonate-containing nucleotides (*e.g.*, methylphosphonate-containing nucleotides are readily available and can be incorporated at any position of an oligonucleotide during synthesis using techniques which are well known in the art.

In essence, the invention contemplates the use of charge-based separation to permit the separation of specific reaction products from the input oligonucleotides in nucleic acid-based detection assays. The foundation of this novel separation technique is the design and use of oligonucleotide probes (typically termed "primers" in the case of PCR) which are "charge balanced" so that upon either cleavage or elongation of the probe it becomes "charge unbalanced," and the specific reaction products may be separated from the input reactants on the basis of the net charge.

In the context of assays which involve the elongation of an oligonucleotide probe (*i.e.*, a primer), such as is the case in PCR, the input primers are designed to carry a net positive charge. Elongation of the short oligonucleotide primer during polymerization will generate PCR products which now carry a net negative charge. The specific reaction products may then easily be separated and concentrated away from the input primers using the charge-based separation technique described herein (the electrodes will be reversed relative to the description in Example 24 as the product to be separated and concentrated after a PCR will carry a negative charge).

VI. Invader™-Directed Cleavage Using Miniprobes And Mid-Range Probes

As discussed in section III above, the Invader™-directed cleavage assay may be performed using inavder and probe oligonucleotides which have a length of about 13-25 nucleotides (typically 20-25 nucleotides). It is also contemplated that the oligonucleotides that span the X, Y and Z regions (see Figure 29), the invader and probe oligonucleotides, may themselves be composed of shorter oligonucleotide sequences that align along a target strand but that are not covalently linked. This is to say that there is a nick in the sugar-phosphate backbone of the composite